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Award Number: W81XWH-04-1-0181

TITLE: Computational Search for Novel Antagonists to the
         Metastatic Mutant Forms of the Androgen Receptor

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REPORT DATE: February 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

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designated by other documentation.

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE February 2005	3. REPORT TYPE AND DATES COVERED Annual Summary (12 Jan 2004 – 11 Jan 2005)

4. TITLE AND SUBTITLE
Computational Search for Novel Antagonists to the Metastatic Mutant Forms of the Androgen Receptor

5. FUNDING NUMBERS
W81XWH-04-1-0181

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Prostate cancer is the most common cancer in the United States and is the second leading cause of cancer death among men in most western countries. The activation of androgen receptor is a major factor contributing to progression of the prostate cancer. Therefore, the administration antiadrogens (androgen antagonists) is a vital part of currently used therapies. However, limited number of available antiandrogens, especially those against mutant forms of androgen receptor found in metastatic prostate cancer, affect successful outcome of a treatment. Major factor impeding discovery of novel antiandrogens was absence of crystal structure of androgen receptor in antagonist conformation. Thus, the first and most important stage of the project involved development and biological validation of the model of interaction of the wild type androgen receptor with known antagonists. This model was developed, and at first validated *in silico* by demonstrating docking selectivity toward known ligands. Then, an extensive ligand database was docked to the model, and the best predicted 16 binders were tested for antagonist properties using transactivation assay in CV1 cells, and their binding to androgen receptor was confirmed by competitive binding assay. From those 16 compounds, one has shown antagonist activity comparable to that of flutamide, clinically used antagonist. The metastatic mutants of the androgen receptor are now being modeled, based on validated model of the wild type receptor.

14. SUBJECT TERMS
Prostate cancer, androgen receptor, antagonists, metastatic mutants

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Prostate cancer is the most common cancer in the United States and is the second leading cause of cancer death among men in western countries. The prostate is important for proper bladder control and normal sexual function in males. The male sex hormone testosterone (belongs to the group of androgen hormones) mainly controls the growth and working of prostate through the androgen receptor (AR) (1). Constitutive activation of AR is also implicated in development and progressing of prostate cancer, especially of metastatic form (2-5). Thus, in addition of surgical removal of prostate gland, androgen ablation therapy is used, which is surgical or chemical castration and/or administration antiandrogens. The antiandrogens, also known as androgen antagonists, are compounds rendering androgen receptor inactive. Unfortunately, the number of clinically available antiandrogens, especially those against mutant forms of androgen receptor found in metastatic prostate cancer, is severely limited. Thus, the major goal of a given research project is to discover novel antiandrogens against metastatic mutant forms of androgen receptor by utilizing the most recent methods of computational biology.

Body

Task 1.

Use protein structure modeling and docking technologies to understand the mechanism of agonist binding and specificity, and to design models of the antagonist bound conformations of AR and its metastatic mutant forms.

a) *Modeling the antagonist conformation of androgen receptor ligand binding domain.*

As of the day this report is being written, the Brookhaven PDB databank does not contain crystal structures for the AR ligand binding domain (LBD) in *antagonist* conformation. The figure below (Fig. 1) displays crystal structures of homologous to androgen receptor, glucocorticoid receptor ligand binding domain in both agonist and antagonist conformations.
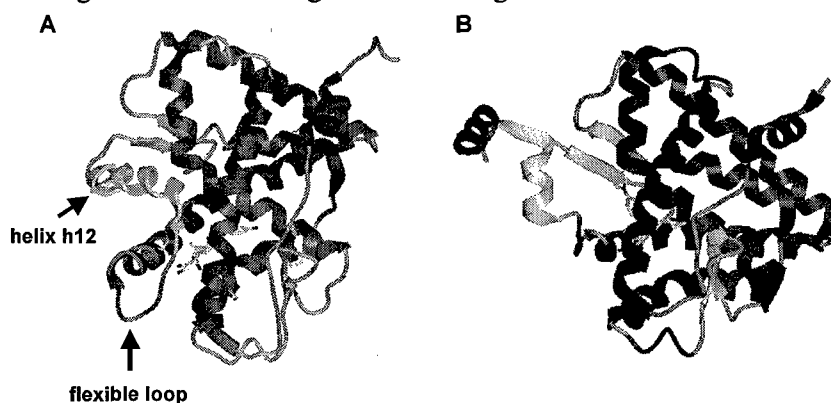

FIGURE 1. **Antagonism phenomenon in glucocorticoid receptor. A.** agonist conformation; **B.** antagonist conformation.

The current view of antagonism phenomenon is that more extended antagonist conformation of the androgen receptor is unable to form transient complex with other nuclear factors, required for transcription initiation (6). The transition to antagonist conformation is mediated by flexible loop (shown in green on Fig. 1) connecting helices 11 and 12, and by helix 12 (shown in yellow on Fig. 1). The flexible loop plays critical role by interacting with ligand. Thus, knowledge of the flexible loop structure in antagonist conformation is absolutely necessary for any virtual ligand screening experiments.

To address this question, comparative protein modeling was applied. Several structures of homologous nuclear receptors were tested as templates. However, docking of known antagonists and their derivatives produced mostly unacceptable results. The binding modes of ligands and their interactions with protein scaffold were not in accordance with published biochemical data. In particular, ligands did not form key interactions with residues, previously shown by site-directed mutagenesis to be essential for ligand binding (7-11).

The analysis of homology models revealed that even though overall fold of all nuclear receptors LBDs is basically identical, the key residues of the androgen receptor LBD were placed into wrong secondary structure elements (e.g. into helix instead of loop and so on). These structural errors resulted mostly from poor sequence alignment due to numerous gaps. To tackle these issues, the best alignment, obtained with glucocorticoid receptor, was corrected manually, and threading-like procedure (as implemented in ICM) was utilized to generate initial approximation of flexible loop and H12 conformations.

The initial model was further refined by iterative cycles of ligand docking, followed by resampling of side chains around docked ligands. The training ligand set consisted of flutamide, hidroxyflutamide, mifepristone and bicalutamide. This procedure was repeated iteratively until acceptable docking scores and ligand binding modes were achieved. From this set of models, two were selected for further development (Fig. 2).
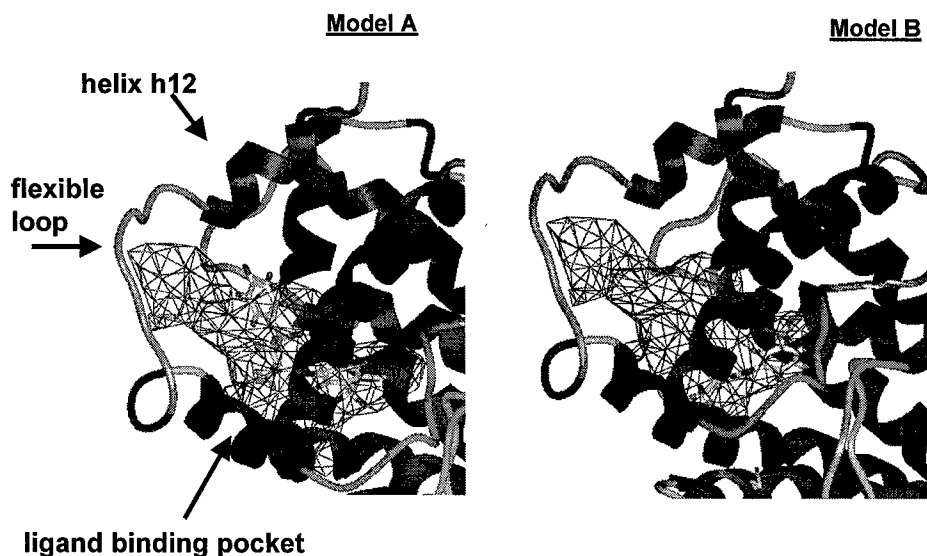

FIGURE 2. **Second generation models of AR LBD in antagonist conformation.** Ligand shown in stick-style are bicalutamide (Model A) and flutamide (Model B).

As seen from the figure, the ligand binding pocket is of sufficient size (≈ 700 A^3) to be able to accommodate chemically diverse set of potential novel binders.

The training and refinement of the second generation models can be explained in the best way by the diagram (Fig. 3).
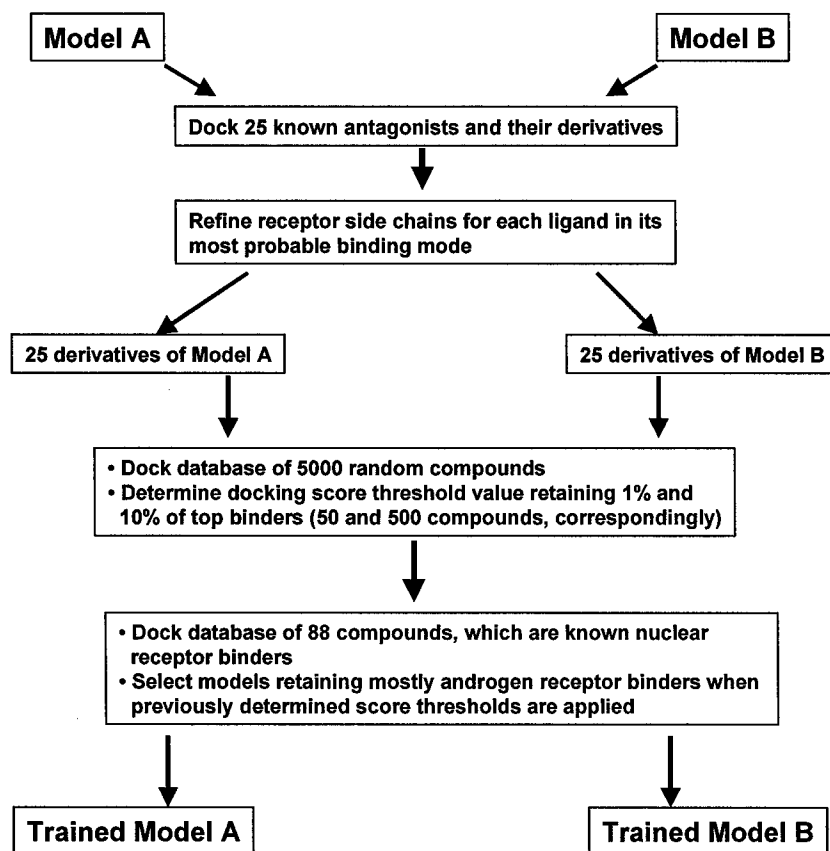

FIGURE 3. **Model training protocol.** Model A was refined with bicalutamide, and Model B with flutamide, respectively. 5000 compounds were randomly selected from ChemDiv database. Database of 88 compounds contained both agonists and antagonists for androgen, estrogen, glucorticoid, progesterone, retinoid, thyroid hormone, retinoic acid, pregnane X receptor and peroxisome proliferator activated receptor.

The described by the diagram procedure was adopted with slight modifications from (12). Briefly, a small database, containing published structures of 25 AR antagonists and their derivatives, was constructed. Each ligand from this database was docked independently into each of the models, generating 50 receptor-ligand complexes. Then, each complex was refined with ligand inside. The database of 5000 compounds, randomly selected from recent ChemDiv database, was docked to each of refined complexes. The results of these docking experiments allowed to determine *sensitivity* of the models, providing 1% and 10% docking scores cutoffs. Then, a database, containing 88 hand-picked agonists and antagonsists toward known nuclear receptors, was docked to each of the refined complexes. These docking experiments allowed to screen for models, based on their *selectivity* toward AR ligands. Both selectivity and sensitivity profiles were used to select "production" models for novel AR antagonist screening. Example of plots, used to select trained

models is shown on Fig. 4.

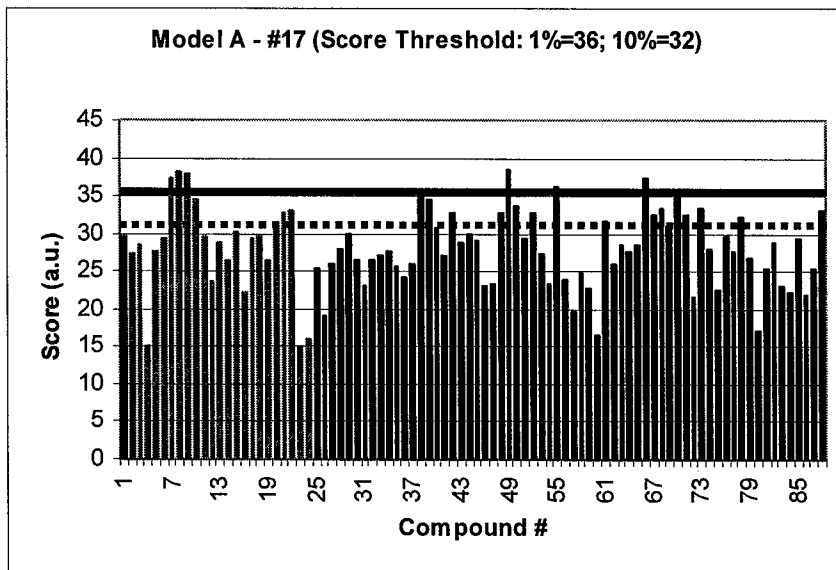**Model A - #17 (Score Threshold: 1%=36; 10%=32)**

FIGURE 4. **Virtual screening results.** The score of known binder (red) and presumed binders (black) is shown. The score thresholds necessary to select 1% (solid line) and 10% (dotted line) of a diverse database of 5000 random compounds are also shown.

It was also determined, that good model selectivity was necessary, but not sufficient. The binding modes of known ligands in some of very selective models were not consistent with available biochemical knowledge. Thus, in addition to selectivity profiles, each model was also examined for proper mode of ligand binding, and preference was given to more meaningful binding over better selectivity.

After all data were analyzed, two best trained models were selected: one from model subset A, and one from model subset B. However, these models had yet to be validated biologically before they could be used as templates for modeling of metastatic androgen mutants. This biological validation had to include *in vitro* transactivation and competitive binding assays with novel potential antagonists.

b) *In vitro biological validation of androgen receptor ligand binding domain in antagonist conformation.*

The KEGG database (13) and the CNS library of ChemBridge were docked into both models. The top binders, scoring above 1% cutoff, were manually inspected and 16 compounds (8 from each model) were purchased. The transactivation CAT assays were performed in collaboration with Prof. Xia Kun Zhang (The Burnham Research Institute), who has kindly provided his lab space, basic reagents and equipment for these experiments. The transactivation experiments were performed as described in *Method* section of this proposal.

Most of the potential ligands did not exhibit either agonist or antagonist effects on the androgen receptor. However, one of the compounds (designated as #11) exhibited antagonist

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activity compared in magnitude to that of flutamide (Fig. 5).

In addition to CAT assays, competitive ligand binding studies have been also performed. These experiments have been done in collaboration with Prof. James T. Dalton (Ohio State University), who kindly agreed to measure competitive binding K_i values with recombinant androgen receptor ligand binding domain.

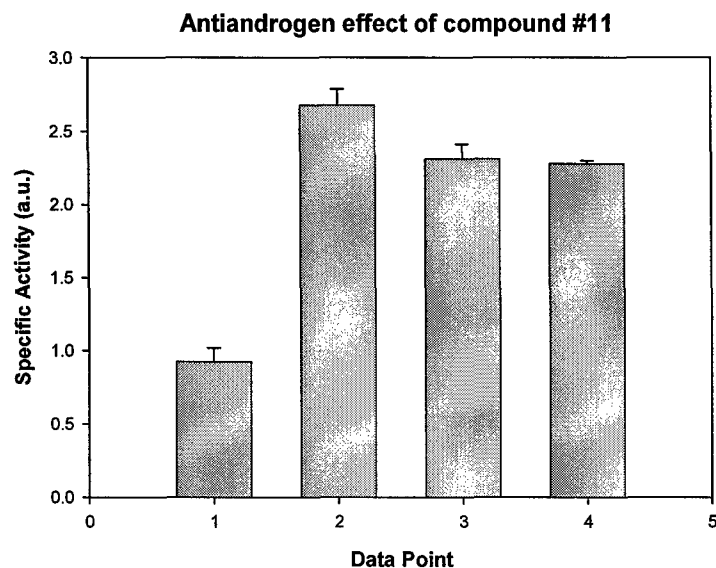**Antiandrogen effect of compound #11**

FIGURE 5. **CAT assay of compound #11.** Bars: 1 – control, 2 - 1.0 nM dehydrotestosterone (DHT), 3 – 1.0 nM DHT + 300.0 nM flutamide, 4 – 1.0 nM DHT + 300.0 nM compound #11.

The results of these experiments are presented in Table 1.

TABLE 1. **Recombinant AR LBD competitive binding assay.**

Compound #	K_i, nM
1	n/a
2	n/a
4	n/a
5	n/a
6	n/a
7	n/a
8	> 1000.0
9	n/a
10	> 2000.0
11	850.0
12	n/a
13	n/a
14	n/a
15	n/a
16	n/a

n/a: no significantly competitive binding was observed; #3: compound was skipped due to extremely poor solubility

The presented data do confirm that antagonist effects of #11 are due to its binding to AR LBD.

Identification of a novel antagonist binder allowed to close on better antagonist models from the set of available 50 models, derived from lines A and B. The ligand #11 was docked into each of 50 models and the best scoring one was selected. Then, the database of available marketed drugs (14) was docked into the selected model together with compound #11 (Fig.6).

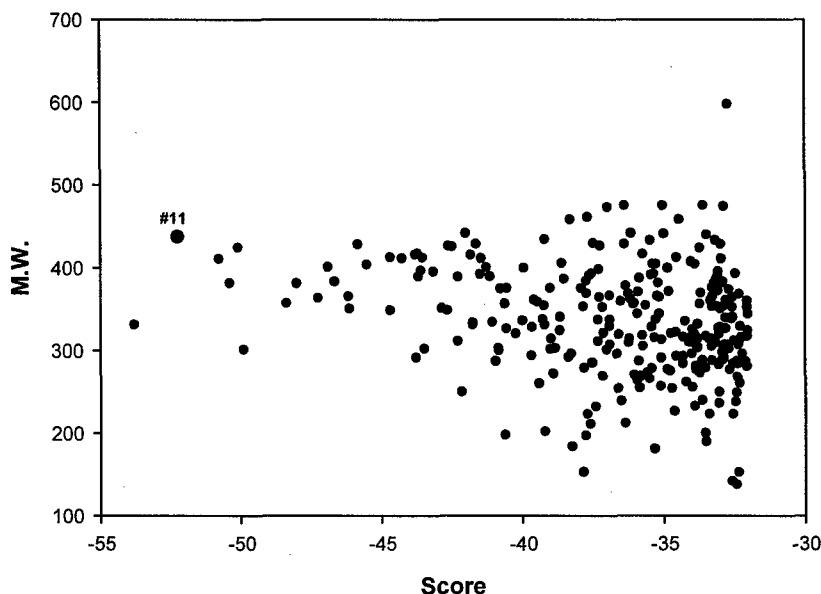Docking of database of marketed drugs

FIGURE 6. **Docking of marketed drugs database into the best scoring model.** Red circle is compound #11; more negative score corresponds to better ligand binding. Default ICM docking score cutoff of – 32 was applied to data. Total of 1729 ligands docked. M.W. – molecular weight.

As seen from the figure, compound #11 scored second place among other ligands. Moreover, compound #11 was one of those marketed drugs. Thus, androgen receptor antagonism activity is its side effect. Side effects among marketed drugs are quiet common, and often beneficial when applied toward cure of a different kind of illness (e.g. Viagra, originally designed to treat hart problems).

Key Research Accomplishments

- Model of the androgen receptor ligand binding domain in antagonist conformation has been built and biologically validated *in vitro*
- Novel non-steroidal antiandrogen against wild type AR LBD was discovered
- Novel antiandrogen is a marketed drug

Reportable Outcomes

A manuscript is currently being prepared for submission to Journal of Medicinal Chemistry.

Conclusions

The most important result of the completed research is biologically validated model of the androgen receptor ligand binding domain in antagonist conformation.

First, this model allowed to identify a novel non-steroidal antiandrogen, which is also an FDA-approved marketed drug. Thus, upon successful *in vivo* validation, this drug could be introduced into clinics. The docking results of marketed drugs database also suggested more compounds for biological testing. These experiments are currently in progress.

Second, biologically validated wild type model provides basis for building models of metastatic mutant forms of androgen receptor. The modeling of mutants and virtual screening for potential antagonist ligands are in progress.

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